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Evaluation of Methodology for Serotyping Invasive and Nasopharyngeal Isolates of *Haemophilus influenzae* in the Ongoing Surveillance in Brazil

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To assess the magnitude of discrepant results obtained by routine *Haemophilus influenzae* serotyping, 258 isolates, collected by the epidemiological surveillance system in Brazil from individuals with invasive diseases or carriage, were evaluated by two slide agglutination (SlAg) methods: SlAg method 1, by which strains were initially screened with a serotype b-specific antiserum, and SlAg method 2, by which strains were tested against all serotype-specific antisera in parallel. Investigators comparing results of the two SlAg methods with those obtained by capsule type-specific PCR were blinded to the method used. The serotype prevalence rates found by the three methods were significantly different, involving discrepancies mainly between serotype b and noncapsulated (NC) isolates. For invasive isolates (n = 131), the overall agreement rate between SlAg method 1 or 2 and PCR was 68.0 or 88.3%, respectively, whereas for colonizing isolates (n = 127) the corresponding rate was 46.5 or 94.2%, respectively. SlAg method 2 improved the ascertainment of serotypes over that obtained with SlAg method 1, demonstrating good correlation with PCR. Use of the polyvalent antiserum as a screening reagent for SlAg for invasive and colonizing isolates showed poor discriminatory power, with a sensitivity of 65.8% and a specificity of 91.7%. We stress the importance of using a well-standardized SlAg methodology and suggest that reference laboratories should utilize PCR routinely to confirm SlAg results and to check all nonspecific SlAg reactions and apparent NC isolates by SlAg in order to provide reliable data on the prevalence of *H. influenzae* serotypes in the *H. influenzae* type b vaccine era.

The development and worldwide implementation of the *Haemophilus influenzae* serotype b (Hib) conjugate vaccine (19) constituted one of the most remarkable public health advances against infectious disease in the past 2 decades. *H. influenzae* remains an important etiological agent of meningitis, septicemia, and pneumonia in infants from countries where the Hib vaccine is not accessible. The decline of infections caused by Hib, especially meningitis, as a result of immunization programs has been notable and well reported (1, 17, 19). Hib vaccine also reduces nasopharyngeal colonization by Hib, thus contributing to indirect protection of unvaccinated infants through herd immunity (3, 4, 8, 14, 24).

Although there has been a remarkable decline in the number of Hib meningitis cases, recent reports have disclosed an emergence of invasive disease caused by non-serotype b or noncapsulated (NC) *H. influenzae* (2, 10, 23, 25, 27). Such disease has been primarily associated with *H. influenzae* localized in respiratory infections, mostly in children above the age of 5 years and adults (16, 29). The reemergence of Hib in vaccinated children has also been reported (15). In Brazil, soon after the first year of vaccine implementation, an increase in serotype a (21) and NC isolates was noticed (data from the Adolfo Lutz Institute [IAL]). These findings reinforce the need to maintain epidemiological surveillance of *H. influenzae* serotypes after the introduction of the Hib vaccine into the routine program in order to evaluate shifts in *H. influenzae* capsule types.

H. influenzae can express six capsule polysaccharides (serotypes a to f), of which serotype b was responsible for the major burden of *H. influenzae* invasive disease before the Hib vaccination era (19, 20, 28, 29, 30). The method most widely used for identifying the capsule serotype of *H. influenzae* is slide agglutination (SlAg) with polyclonal sera. However, misidentifications of *H. influenzae* serotypes by this method have been reported (7, 22, 27) and recently have become a matter of concern (13), attributed to the performance of the assay and difficulties in the interpretation of SlAg reactions. A short time ago, preliminary results during surveillance for *H. influenzae* from the nasopharynx in a vaccinated population in Brazil showed a high rate of serotype b among colonizing (Col) *H. influenzae* isolates. This amazing outcome was attributed to cross-agglutinations and equivocal interpretations of the reaction. Thus, to ascertain the magnitude of the discrepancy in routine *H. influenzae* serotyping, we used isolates collected during the national epidemiological surveillance in Brazil to compare results of serotyping by two SlAg methods with those of capsule typing performed by PCR (6). The use of a polyvalent antiserum as a screening reagent for slide agglutination was also evaluated.

MATERIALS AND METHODS

In Brazil, invasive (Inv) *H. influenzae* isolates are routinely isolated by public health laboratories and institutions and then submitted to the IAL, located in the

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city of São Paulo, as part of the national epidemiological surveillance program. From 1985 through 2002, the IAL received 3,632 invasive *H. influenzae* isolates from blood, cerebrospinal fluid, or pleural fluid for confirmation of species identification and serotyping. Isolates came from the southeastern (52%), northeastern (25%), central west (14%), and southern (9%) regions of the country. Additionally, 338 *H. influenzae* carriage isolates were recovered during investigations on *H. influenzae* colonization of healthy children from 2000 to 2002. All strains were kept lyophilized, and the respective demographic information was stored in a database.

To enhance the feasibility of selecting the isolates, a convenience sampling (11) was used to select a total of 258 *H. influenzae* isolates for this study, including 131 Inv isolates and 127 nasopharyngeal Col isolates. It was estimated that a sample size of around 130 isolates would be required to detect at least 10% in capsule type results between SlAg methods and PCR, with a 5% error. The isolates were blindly tested by three methods (SlAg method 1, SlAg method 2, and PCR).

Standard strains for all serotypes (ATCC 9006, ATCC 35533, ATCC 9007, ATCC 9332, ATCC 8142, and ATCC 9833) and an NC strain (ATCC 49247) were used as positive controls for SlAg tests and PCR. The strains were subcultured onto brain heart infusion chocolate agar (Difco BD Bioscience, Cockeysville, Md.) containing 10% horse blood at 37°C for 18 h, and the bacterial growth was used for both SlAg and PCR techniques.

SlAg. Each isolate was pretested with a formalinized 0.85% NaCl solution (saline) to prevent autoagglutination. Monovalent serotype-specific rabbit antisera (specific for serotypes a through f) reconstituted at IAL and obtained from Difco BD Bioscience were utilized. The set of antiserum reagents was tested with quality control standard strains before use. SlAg was performed by two methods. Method 1 uses a serotype b-specific antiserum for screening, and method 2 runs all antisera in parallel. SlAg method 1 was performed according to the manufacturer's instructions, entitled "Serological identification of *Haemophilus influenzae*," by transferring a loopful of bacterial growth to 50 μl of a serotype b-specific antiserum, suspending thoroughly, and rocking the slide for 1 min. By this method, the serotype b-specific antiserum was used for the first screening, and a positive agglutination result was considered to indicate serotype b. If the isolate was nonreactive with the serotype b-specific antiserum, it was tested with the remaining antisera (specific for serotypes a and c to f). If a negative reaction occurred with all the antisera in the set, the strain was considered NC. SlAg method 2 was performed by transferring 10 μl of a milky suspension of the bacterial cells made in 0.85% formalinized saline to 10 μl of the antiserum; all specific antisera were run in parallel, and the slide was rocked for 1 min. For both SlAg methods, the intensity of the SlAg reaction was recorded by using symbols representing the absence or different grade of agglutination (−, negative; +, slow and weak; ++++, rapid, with formation of large clumps). Positive agglutination was defined as the occurrence of large clumps (+++ or ++++) with only one specific antiserum. The isolate was considered NC when negative agglutination results with a complete set of antisera or weak reactions with at least two antisera (nonspecific reaction) were observed. When positive agglutination occurred with at least two specific antisera, the isolate was considered polyagglutinated.

Evaluation of the *H. influenzae* polyvalent antiserum as a screening reagent for SlAg. A polyvalent antiserum from Difco was tested with 62 *H. influenzae* isolates (30 Inv and 32 Col isolates) by mixing 10 μl of a milky suspension of the bacterial cells with 10 μl of the antiserum. The presence of large clumps was considered to indicate positive agglutination.

PCR assay. DNA suspensions from isolates were prepared by transferring four to six colonies from overnight culture onto a brain heart infusion chocolate agar plate in 60 μl of sterile tissue culture water. This suspension was boiled for 10 min and centrifuged at 1,300 rpm (on a Marathon 26kmr instrument [Fischer Scientific]) for 3 to 5 min. The supernatant was stored at −20°C until testing. PCR was performed by using oligonucleotide primers synthesized based on the published DNA sequences for the *bexA* gene (the gene for capsule expression), which distinguishes capsulated from NC *H. influenzae* isolates, and for all six capsule type-specific genes (6). The PCR mixture (25 μl) contained PCR buffer (Gibco BRL Life Technologies), 200 μM deoxyribonucleotides, 1 μM each oligonucleotide primer (Gibco BRL Life Technologies), and 0.5 U of *Taq* DNA polymerase (Gibco BRL Life Technologies). A 1-μl volume of DNA suspension was used as the template. DNA amplification (Perkin-Elmer, Norwalk, Conn.) was carried out as described previously (6), but 55°C was used as the annealing temperature. One primer set derived from the sequence for the gene coding for outer membrane lipoprotein P6, which is present in both capsulated and NC *Haemophilus* strains, was also used as a control for PCRs (26). PCR products were resolved by electrophoresis (Gibco BRL Life Technologies) on a 1% agarose gel (Sigma Chemical Co., St. Louis, Mo.) for 1 h at 100 V. A 100-bp molecular weight marker ladder (Gibco BRL Life Technologies) was included in all gel electrophoreses. Gels were stained with ethidium bromide and photographed under UV light.

A second round of PCR was performed with the primary PCR product to confirm negative amplification at the first round. For this purpose, a third internal primer and one of the primers from the first round of PCR were used under the same PCR conditions as those for the first round (6). Capsule typing was considered positive when DNA amplification of the *bexA* gene and one of the specific capsule genes occurred; the strain was typed as a capsule expression mutant when PCR results were negative for the *bexA* gene and positive for a specific capsule gene; the isolate was determined to be NC when neither the *bexA* gene nor a specific capsule gene was amplified by PCR (6).

Data analysis. The results provided by SlAg method 1, SlA method 2, and PCR were tested in a blind manner and analyzed separately for Inv and Col isolates. The prevalences of capsule types and their respective 95% confidence intervals (95% CI) were estimated for each method. Differences in prevalence between the methods were considered significant if the 95% CI did not overlap. PCR was taken as a reference method for comparison purposes. The agreement rate between PCR and each SlAg method was calculated as the number of isolates with concordant results for a given capsule type by PCR and the SlAg method divided by the total number of isolates with that serotype result by the SlAg method. Agreement rates were assessed separately for the Inv and Col isolate groups. A result of NC by SlAg and a corresponding capsule type b^- result by PCR (loss of the gene for capsule expression [12]) were considered concordant. Because the auto- and polyagglutination characteristics do not match comparative results, isolates belonging to these groups were not included in calculating agreement between SlAg method 2 and PCR. To evaluate the performance of the polyvalent antiserum as a screening reagent for the SlAg method, the results obtained by this screening test were compared with the respective SlAg method 2 results.

RESULTS

The prevalences of capsule types among *H. influenzae* Inv isolates by the three methods studied are shown in Table 1. Significant differences in the prevalence rates were found for capsule type b by SlAg method 1 (12.1%; 95% CI, 7.4 to 18.7%) versus PCR (29.8%; 95% CI, 22.4 to 38.0%) and for NC isolates by SlAg method 1 (52.0%; 95% CI, 43.3 to 60.4%) versus PCR (28.3%; 95% CI, 21.0 to 36.4%). No statistical difference was detected between SlAg method 2 and PCR. Isolates of serotypes a, c, d, e, and f were identified at equal or similar rates by the three methods. Three isolates showed polyagglutination by SlAg method 2; of these, two were recognized as NC and one was recognized as type b by PCR and SlAg method 1. PCR distinguished four capsule type b^- isolates that were correctly serotyped as NC by both SlAg methods. The autoagglutination reaction was not detected among the Inv isolates.

The overall agreement rate for Inv isolates between SlAg method 1 and PCR was 68.0% (Table 1). A 20.3% increase in the agreement rate was observed when SlAg method 2 was used. The low agreement rates for NC isolates were noteworthy, since only 47% of isolates identified as NC by SlAg method 1 and 71% so identified by SlAg method 2 were confirmed by PCR. Those isolates falsely identified as NC by SlAg method 1 were recognized as types a (n = 9), b (n = 22), and f (n = 1) by PCR, while those isolates falsely identified as NC by SlAg method 2 were recognized as types a (n = 2) and b (n = 9) by PCR. Analysis of the intensity of the agglutination reactions obtained with SlAg methods 1 and 2 for these groups of isolates showed a tendency to weak reactions (+), leading to misidentification of serotype results. Non-b capsulated strains were well characterized by both SlAg methods, with agreement rates ranging from 80.0 to 100.0%, except for two isolates

TABLE 1. Comparison of frequencies of capsule types for 131 *H. influenzae* invasive isolates according to SlAg method 1, SlAg method 2, and PCR

H. influenzae capsule type	No. (%) of isolates with the indicated capsule type by:			No. (%) of isolates for which PCR results agree with:	
	SlAg method 1	SlAg method 2	PCR	SlAg method 1	SlAg method 2
a	34 (26.0)	39 (29.8)	39 (29.8)	28 (82.3)	36 (90.0)
b	16 (12.1)	28 (21.5)	39 (29.8)	14 (87.5)	27 (96.4)
c	2 (1.5)	2 (1.5)	2 (1.5)	2 (100.0)	2 (100.0)
d	4 (3.0)	2 (1.5)	2 (1.5)	2 (50.0)	2 (100.0)
e	1 (0.8)	1 (0.8)	1 (0.8)	1 (100.0)	1 (100.0)
f	6 (4.6)	7 (5.3)	7 (5.3)	6 (100.0)	7 (100.0)
NC	68 (52.0)	49 (37.4)	37 (28.3)	32 (47.0)	34 (71.0)
b^{-}[a]	NA[b]	NA	4 (3.0)	4 (100.0)[c]	4 (100.0)[d]
Polyagglutination	0	3 (2.3)	P[e]	NA	NA
Total isolates	131	131	131	89 (68.0)	113 (88.3)[f]

[a] Capsule expression mutant.
[b] NA, not applicable.
[c] Four isolates identified as b^{-} by PCR and as NC by SlAg method 1 were considered concordant.
[d] Four isolates identified as b^{-} by PCR and as NC by SlAg method 2 were considered concordant.
[e] Three isolates identified as polyagglutinating by SlAg method 2 were identified as NC (n = 2) and type b (n = 1) by PCR.
[f] Three polyagglutinated isolates were excluded from calculation of the agreement rate.

identified as serotype d by SlAg method 1, which were capsule type d and NC by PCR.

The prevalences of capsule types among Col isolates according to SlAg method 1, SlAg method 2, and PCR are shown in Table 2. Prevalence rates for type b were significantly different by SlAg method 1 (41.0%; 95% CI, 32.6 to 49.6%) versus SlAg method 2 (2.3%; 95% CI, 0.6 to 6.3%) and PCR (2.3%; 95% CI, 0.6 to 6.3%). Also, divergent rates of NC isolates were identified by SlAg method 1 (41.0%; 95% CI, 32.6 to 49.6%) compared with SlAg method 2 (86.1%; 95% CI, 78.9 to 91.1%) and PCR (93.0%; 95% CI, 87.4 to 96.5%). Serotypes a, c, d, e, and f were identified at low prevalences or were not identified depending on the method used. Three isolates identified as polyagglutinated and three identified as autoagglutinated by SlAg method 2 were typed as NC by PCR. Among Col isolates, none was identified as type b^{-} by PCR.

The overall rate of agreement in capsule typing of *H. influenzae* Col isolates between SlAg method 1 or 2 and PCR was 46.5 or 94.2%, respectively (Table 2). Thus, a notable increase of 47.7% in the agreement rate was achieved by using SlAg method 2. Comparison of the findings by SlAg method 1 and PCR showed that all 52 isolates serotyped as NC and the two serotype e isolates were confirmed by PCR. However, SlAg method 1 produced 68 false-positive serotype results, leading to low agreement rates, ranging from 0 to 27.3%. Of these, the most important discrepant results were the 50 isolates falsely identified as serotype b by SlAg method 1; by PCR, 49 of those were identified as NC and 1 was identified as type a. The isolates falsely identified as serotypes a (n = 4), c (n = 3), d (n = 3), and f (n = 8) by SlAg method 1 were NC (n = 17) and type b (n = 1) by PCR. Analysis of the intensity of the agglutination reactions of these 50 false serotype b isolates by SlAg method 1 revealed that most of these isolates presented weak reactions (+ or ++). When the results of SlAg method 2 and PCR were compared, only 7 discrepant capsule types were found: specifically, isolates identified as serotypes a (n = 2), b

TABLE 2. Comparison of frequencies of capsule types for 127 *H. influenzae* nasopharyngeal isolates according to SlAg method 1, SlAg method 2, and PCR

H. influenzae capsule type	No. (%) of isolates with the indicated capsule type by:			No. (%) of isolates for which PCR results agree with:	
	SlAg method 1	SlAg method 2	PCR	SlAg method 1	SlAg method 2
a	4 (3.1)	3 (2.3)	1 (0.8)	0 (0)	1 (33.4)
b	52 (41.0)	3 (2.3)	3 (2.3)	2 (3.8)	2 (66.7)
c	3 (2.3)	2 (1.6)	0	0 (0)	0 (0)
d	3 (2.3)	0	0	0 (0)	0 (100.0)
e	2 (1.6)	1 (0.8)	2 (1.6)	2 (100.0)	1 (50.0)
f	11 (8.7)	3 (2.3)	3 (2.3)	3 (27.3)	3 (100.0)
NC	52 (41.0)	109 (86.1)	118 (93.0)	52 (100.0)	107 (90.7)
Polyagglutination	0	3 (2.3)	P[a]	NA[b]	NA
Autoagglutination	0	3 (2.3)	A[c]	NA	NA
Total isolates	127	127	127	59 (46.5)	114 (94.2)[d]

[a] Three isolates identified as polyagglutinating by SlAg method 2 were identified as NC by PCR.
[b] NA, not applicable.
[c] Three isolates identified as autoagglutinating by SlAg method 2 were identified as NC by PCR.
[d] Six isolates (poly- and autoagglutinated) were excluded from calculation of the agreement rate.

(n = 1), c (n = 2), and NC (n = 2) by SlAg method 2 were typed as NC (n = 5) and types b (n = 1) and e (n = 1) by PCR (Table 2).

Analysis of the intensity of the agglutination reactions disclosed that 11.0% of Inv isolates and 14% of Col isolates presented nonspecific reactions. In general, positive agglutinations for Inv isolates presented large clumps (+++ and ++++), while for Col isolates, positive reactions showed a less distinct pattern of agglutination.

Evaluation of the screening test with a polyvalent antiserum compared with the respective SlAg method 2 detected a concordance rate of 75.8%(95% CI, 64.0 to 85.2%); 8.3% (95% CI, 1.4 to 24.9%) of isolates presented false-positive agglutination and 34.2% (95% CI, 20.5 to 50.2%) presented false-negative agglutination by the screening test. This evaluation of the polyvalent antiserum showed no difference between Inv and Col isolates.

DISCUSSION

This study demonstrates the limitations of SlAg methods for *H. influenzae* serotyping by comparison with capsule type results provided by PCR. The majority of equivocal agglutination reaction results were due to nonspecific agglutination or cross-reactions among the serotype-specific antisera. Therefore, substantial misinterpretation may occur when SlAg is not properly applied.

The use of a type b-specific antiserum for the first screening (SlAg method 1) correctly identified only 68.0% of Inv isolates and 46.5% of Col isolates. For Inv isolates, the most important misclassification was the overdiagnosis of NC isolates that were confirmed as type b by PCR. Thus, SlAg method 1 underestimated by approximately 18.0% the prevalence of serotype b isolates among Inv isolates. On the other hand, for Col isolates, SlAg method 1 overestimated the prevalence of serotype b by approximately 39.0%. This excess of serotype b isolates was identified as NC by PCR, which explains the poor agreement between SlAg method 1 and PCR (3.8%) for serotype b. Therefore, the largest part of the discrepant results found by SlAg method 1 involved NC and serotype b isolates. However, the use of all antisera in parallel (SlAg method 2) significantly improved the overall capsule type agreement with PCR for both Inv and Col isolates, by approximately 20.0 and 48.0%, respectively. In fact, SlAg method 2 has the visual advantage of pointing out the pattern of the agglutination with all six type-specific-antisera, making interpretation of serotyping results easier and more straightforward. A small proportion of incorrect serotype identifications still occurred, even when all six type-specific antisera were used. This may be related to the individual characteristics of expression of capsule and/or other antigens on the bacterial surface (9).

The use of a polyvalent antiserum as a screening reagent in SlAg demonstrated poor discriminatory power, with a low concordance rate of approximately 76.0%. The weak performance of this screening test had been reported previously (22).

In a recent report on discrepancies in *H. influenzae* serotyping among Inv isolates, the authors stated that the main diagnostic error was the underestimation of NC isolates, which yielded false-positive serotype b results (13). This mistake was attributed to the unique use of a type b-specific antiserum for screening, leading one to consider any form of agglutination to be an *H. influenzae* type b-positive reaction (13). In the present study, the weak intensity of the agglutination reaction explains the false identification of 50 Col isolates as serotype b by SlAg method 1.

Our observation that nasopharyngeal *H. influenzae* isolates were more challenging to serotype than *H. influenzae* Inv isolates is supported by the higher prevalence of NC isolates observed in carriage, with a high diversity of antigens on the bacterial surface. The relatively lower production of capsule among capsulated strains (9, 16, 18) may result in a larger number of nonspecific agglutination reactions than is obtained with *H. influenzae* Inv isolates.

Another important point observed in this study relates to the bacterial inoculum for SlAg. The use of bacterial growth directly transferred to the antiserum by means of the bacteriological loop (SlAg method 1), in accordance with the manufacturer's instructions, may increase the misreading of the agglutination reaction, because a different inoculum of bacteria may be used in each reaction. Also, the bacteria may not be completely emulsified in the antiserum, because this step is time-consuming. Thus, a bacterial suspension in saline (SlAg method 2) is more appropriate for performance of SlAg.

These misleading results of *H. influenzae* serotyping among Inv and Col isolates are of concern, especially for isolates collected in the postvaccine period. Many countries have been assessing the effectiveness of the *H. influenzae* conjugate vaccine based on serotyping results for isolates recovered from surveillance systems and on the effect of the vaccine on nasopharyngeal carriage. The potential serotype discrepancies generated by SlAg suggest that the burden of Hib disease as well as Hib carriage status may be improperly estimated, producing distortions in the assessment of the impact of the *H. influenzae* conjugate vaccine.

Serotyping has been and is currently used for *H. influenzae* because it is simple and rapid to perform and is a valuable tool for epidemiological studies. Therefore, it is crucial to have a well-standardized SlAg in order to assess the actual prevalence of *H. influenzae* serotypes and also to allow comparisons between studies. Prudence during SlAg execution is needed; it is essential always to apply saline and all serotype-specific antisera in parallel. The present study found that this procedure achieved good accuracy compared with PCR.

SlAg method 1 uses a large volume of antiserum and is therefore unnecessarily expensive. If the cost of antiserum is an issue, an alternative approach would be to routinely test the isolate with saline, a type b-specific antiserum, and one other antiserum, specific for the next most prevalent type in the country. If there is agglutination with the type b-specific antiserum and no reaction with the second monospecific antiserum, the strain is a presumptive type b and this result should be confirmed by PCR; if there is no agglutination with both monospecific antisera, then further antisera should be tested. Capsule typing by PCR is easy to perform and provides an objective interpretation but is not as rapid as SlAg. PCR reliably resolves any misinterpretation in SlAgs and provides the capsule type of auto- and polyagglutinated strains. In addition, PCR recognizes capsule-deficient mutant isolates, providing new insights into the pathogenesis of infections caused by *H. influenzae* in the vaccination period (12, 18).

In view of these observations, when a nonspecific reaction or an NC result is obtained by SlAg, reference laboratories should routinely employ PCR as a complementary tool for *H. influenzae* serotyping. The PHLS *Haemophilus* Reference Unit, Oxford, United Kingdom, has included this approach in the Quality Assurance Program for the SIREVA-Vigia Latin America surveillance group (5). With the worldwide use of the Hib conjugate vaccine, a new epidemiological scenario of the diseases caused by *H. influenzae* has been established. In this environment, reliable *H. influenzae* capsule identification is fundamental for monitoring the potential changes in the prevalences of *H. influenzae* serotypes and for accurately estimating the impact of the Hib vaccine through the years.

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